

**SCREENING OF *UGT1A1* GENE AND GENOTYPE-PHENOTYPE  
CORRELATIONSHIP IN NEONATAL JAUNDICE FROM A SAMPLE OF  
NEWBORNS IN KELANTAN**

**by**

**NUR HASNAH BINTI MA'AMOR**

**Thesis submitted in fulfilment of the requirements  
for the degree of  
Master of Science**

**UNIVERSITI SAINS MALAYSIA**

**APRIL 2012**

**PENYARINGAN *UGT1A1* GEN DAN HUBUNGKAIT GENOTIP-  
PENOTIPIK DALAM PENYAKIT KUNING DARIPADA SAMPLE BAYI  
BARU LAHIR DI KELANTAN**

**oleh**

**NUR HASNAH BINTI MA'AMOR**

**Tesis yang diserahkan untuk  
memenuhi keperluan bagi  
Ijazah Sarjana Sains**

**UNIVERSITI SAINS MALAYSIA**

**APRIL 2012**

## **ACKNOWLEDGEMENTS**

Firstly, praise is to Allah S.W.T for giving me strength, good health, motivation, and patience in completing my master's research. I would like to express my sincere appreciation and the deepest gratitude to my beloved and supportive supervisor, Prof Hans Van Rostenberghe, a lecturer, head department and neonatologist in Paediatric Department, Health Campus, Universiti Sains Malaysia, Kelantan for his patience and guidance along the journey to complete my master research. Special thanks to both my co-supervisors, Prof Madya Dr Narazah Mohd Yusoff, a lecturer in Advance Medical and Dental Institute (IPPT) and Prof Madya Dr Zilfalil Alwi, a lecturer in paediatric Department, Health Campus, Universiti Sains Malaysia Kelantan for their help and guidance.

Special thanks to Ministry of Science and Technology Innovations or MOSTI (305/PPSP/6113605 or 06-01-05-SF0166) and also Student Incentive Grant (1001/PPSP/8122030) for their financial support to complete this study. Not forgetting IPS, USM for awarding me the Fellowship scheme for financial support.

I would like to express my deepest gratitude to all parents for their time, patience and cooperation in allowing their neonates to take part in this study. Thanks to the staff in 1 Mutiara, 1 Timur Belakang (1 TB) and 1 Nilam (Neonatal Intensive Care Unit, NICU), the 2 doctors in Master of Medicine, Dr Fazlida and Dr Lilian, and Mardina, Research Assistant for MOSTI's project for her cooperation by helping me either in collecting the subjects and data, or giving guidance.

To all the staff in Human Genome Centre, PPSP, Universiti Sains Malaysia, thanks for the time and cooperation in the whole process of my study. A deep appreciation

goes to my colleagues and friends: Ain, Oh, Che Wan, Aini, Along, Muni, Shikin, Wana, Kak Adiya, Kak Lin, Mar, Atif, Yan Yan, Rani, Fatemeh, Iman, Azi, Amin, Syibli, Tasya, Kila, Abang Nizam, Arif, Kak Sha, Kak Mareen, Arfah, Siti, Huda, Dr Alyaa, Ina, Sathiya, Loo, Au, Kak Hatin, Aizat and others for their cooperation, support, and time. Special thanks to Dr Surini Yusoff, a lecturer from Paediatrics Department, PPSP, USM for her critics, guidance, ideas, time and support in completing my master project. Not forget to Dr Andrew J.Cassidy as his kindly permission to use one of his article's figures (figure 1.3 page 20).

A deep gratitude to my beloved parents and siblings for their support, prayers and cooperation: En Ma'amor bin Kamit and Puan Raudzah binti Mastor for allowing me to further my study by doing my master in USM, Kelantan. Lastly, thanks to all who directly or indirectly contributed in this study. May God bless you.

## TABLE OF CONTENTS

	PAGE
Acknowledgements	iii
Table of contents	v
List of tables	ix
List of figures	xi
List of plates	xii
List of appendices	xiii
List of abbreviations	xiv
List of symbols	xviii
Abstrak	xix
Abstract	xxi
<b>CHAPTER 1 INTRODUCTION</b>	
1.1 Background of the study	1
1.2 Definition of Neonatal jaundice (NNJ)	3
1.3 Bilirubin metabolism	4
1.4 Clinical aspects of neonatal jaundice (NNJ)	
1.4.1 Unconjugated bilirubin (Indirect hyperbilirubinemia)	8
1.4.2 Conjugated bilirubin (Direct hyperbilirubinemia)	8
1.5 Common cause of neonatal jaundice	
1.5.1 Physiological jaundice	9
1.5.2 Pathological jaundice	9
1.5.2.1 Hemolytic factors	
1.5.2.1(i) Glucose-6-phosphate dehydrogenase (G6PD)	
Deficiency	10
1.5.2.1(ii) ABO incompatibility	11
1.5.2.2 Non-hemolytic factors	11
1.6 Clinical syndromes associated with mutation in the <i>UGT1A1</i> gene	15
1.6.1 Gilbert syndrome (GS)	15
1.6.2 Crigler-Najjar syndrome (CN)	17

1.6.2 (i) Crigler-Najjar syndrome Type I (CNI)	17
1.6.2 (ii) Crigler-Najjar syndrome Type II (CNII)	18
1.7 Uridine diphosphoglucuronate glucuronosyl transferase (UDPGT) 1A	19
1.8 Bilirubin uridine diphosphate-glucuronosyltransferase 1A1 ( <i>UGT1A1</i> )	
Gene	21
1.9 Mutation in the <i>UGT1A1</i> gene	23
1.10 Single nucleotide polymorphism (SNP) in <i>UGT1A1</i> gene	26
1.11 Technique used for identification of mutations in the <i>UGT1A1</i> gene	30
1.12 Denaturing high performance liquid chromatography (DHPLC)	33
1.12.1 Principles of DHPLC	34
1.12.2 Performance of DHPLC	36
1.13 Objectives	38
1.13.1 Aim of study	38
1.13.2 Specific objective	38

## CHAPTER 2 MATERIALS & METHODS

2.1 Setting	39
2.2 Study design	39
2.3 Subjects	39
2.4 Collection of blood samples	40
2.5 Association of genotype with relevant phenotypic traits	44
2.6 Sample size	44
2.7 Laboratory procedures	
2.7.1 Materials used in the lab for this study	
2.7.1.1 DNA extraction kit	45
a. Qiagen Protease stock solution	46
b. Buffer AW1 & Buffer AW2 (Washing buffer)	46
c. Buffer AL (Lyses buffer)	46
d. Buffer AE	46
e. QIAamp spin columns	47
2.7.1.2 Polymerase chain reaction (PCR) amplification	
a. 10x PCR buffer II	47
b. 10mM dNTP blend	47
c. 25mM MgCl <sub>2</sub>	47
d. 5µl Amplitaq gold DNA polymerase (250 units)	47
e. Primers	48
2.7.1.3 Gel electrophoresis	
a. Agarose powder	51
b. Tris base EDTA (TBE) buffer	51
c. 6x blue/orange loading buffer/loading dye	51
d. SYBR stain (SYBR®Green I)	52
e. DNA marker	52
2.7.1.4 Denaturing high performance liquid chromatography (DHPLC) analysis	
(A) Modules	
(a) Autosampler (ProStar 430)	53

(b) Oven (Prostar 520)	53
(c) Ultraviolet (UV) detector (ProStar 340)	53
(d) Pump A and B (ProStar 210)	54
(e) Star software	54
(B) Helix analysis column	55
(C) DHPLC reagents	
(a) Buffer A	55
(b) Buffer B	55
(c) Acetonitrile (CAN), CH <sub>3</sub> CN	55
(d) Isopropanol (C <sub>3</sub> H <sub>8</sub> O)	56
(e) Puc 18	56
(f) DYS 271	56
2.7.1.5 DNA sequencing	
(A) PCR purification reagents	57
(a) Buffer PB	57
(b) Buffer PE	57
(c) Buffer EB	57
(d) Spin column	58
(B) Cycle sequencing reagent	
(a) 5X sequencing buffer	58
(b) Big dye® Terminator V3.1 cycle sequencing kit	58
(c) Primers (forward & reverse)	58
(d) DNA templet	58
(C) DNA/EDTA precipitation reagents	
(a) EDTA solution	59
(b) Absolute ethanol and 70% ethanol	59
(c) Hi-Di™ formamide	59
2.7.2. Laboratory methods used in this study	
2.7.2.1 DNA extraction	60
2.7.2.2 DNA quantification	
(a) NanoQuant	61
2.7.2.3 Polymerase chain reaction (PCR) amplification	
(a) Primer dilution	62
(b) PCR protocol	62
(c) PCR reaction & condition	63
(d) 2% agarose gel electrophoresis	68
2.7.2.4 Mutational analysis by DHPLC	
(a) Melting temperature prediction	68
(b) Initiation of process	69
(c) Analysis procedure	
(c i) Validation of system procedure	70
(c ii) Temperature mapping	74
(c iii) Slow Re-annealing	76
(c iv) Sample analysis procedures	76
2.7.2.5 DNA sequencing	
(a) PCR purification protocol	77
(b) Cycle sequencing	78
(c) EDTA/Ethanol precipitation	81
2.7.2.6 Data Analysis	81

## CHAPTER 3 RESULTS

3.1 Baseline data	
3.1.1 Gestational age and birth weight	82
3.1.2 Age of the subjects	84
3.1.3 Sex	84
3.1.4 Family history	87
3.1.5 Commonly identified risk factors for neonatal jaundice	87
3.2 Isolation and amplification of DNA	
3.2.1 DNA quantification	89
3.2.2 PCR amplification in whole exons (exon 1 to exon 5) of <i>UGT1A1</i> gene	89
3.3 DHPLC screening mutation analysis	92
3.4 DNA sequencing analysis	95
3.5 Summary the findings of screening for mutation in the exons of the <i>UGT1A1</i> gene in Malay population	105
3.6 Phenotypic correlation of found variant genotypes	107

## CHAPTER 4 DISSCUSSION

4.1. Demographic and Clinical Data	110
4.2 The G71R (c.211G>A) mutation	
4.2.1 G71R mutation: frequency	111
4.2.2 G71R mutation: relationship with jaundice	113
4.3 Other variants in the exons of the <i>UGT1A1</i> genes	
(a) c.774 T>A (S258R)	115
(b) c.686 C>A, (P229Q)	115
(c) c.1092 G>A (P364P)	117
(d) c.1234 A>T (T412S)	118
(e) c.1091 C>T (P364L)	118
4.4 Other variants in the introns of the <i>UGT1A1</i> genes	119
(a) IVS2-82 T>C	119
(b) IVS2-86 C>T	120
(c) IVS2+15 T>C	120
4.5 Genotypic-phenotypic correlation	120
4.6 Primers design in the exon 1 of the <i>UGT1A1</i> gene in neonatal jaundice	121
4.7 Another isoform group in UGT1A	122
4.8 Limitations of this study	122
4.9 Strengths of the study	123
4.10 Implications for future research	124

CHAPTER 5 CONCLUSION	127
----------------------	-----

REFERENCESS	128
-------------	-----

APPENDICES	138
------------	-----



## List of Tables

Table		Page
Table 1.1	The factors associated with neonatal jaundice	13
Table 1.2	Mutations identified in subjects with CN I syndrome	28
Table 1.3	Mutations in the <i>UGT1A1</i> coding region	29
Table 2.1	Oligonucleotide primers for PCR amplifications of <i>UGT1A1</i> gene	49
Table 2.2	Preparation of PCR master mix (16µl- final total volume) for jaundice & control samples in each primers in <i>UGT1A1</i> gene	64
Table 2.3	Preparation of PCR master mix (30µl- final total volume) Of wild type for each primer in <i>UGT1A1</i> gen	65
Table 2.4	Condition used in the PCR amplification process	66
Table 2.5	Specific annealing temperatures (PCR amplification)	67
Table 2.6	Optimum temperatures DHPLC analysis in	75
Table 2.7	List of Reagent and Volume in cycle sequencing	79
Table 2.8	Condition in the thermo cycler machine	80
Table 3.1	Comparison in between two groups in gestational age and birth weight	83
Table 3.2	G6PD deficiency, ABO incompatibility and DCT results in jaundice group and control group	87
Table 3.3	Genotype distribution and allele frequency of mutations and SNPs among jaundice and control in <i>UGT1A1</i> gene	101
Table 3.4	List of subjects (jaundice) with the mutations or SNPs in exon of <i>UGT1A1</i> gene	102
Table 3.5	List of subjects (control) with the mutations or SNPs in exon of <i>UGT1A1</i> gene	103
Table 3.6	Final finding observed of <i>UGT1A1</i> gene in this study	106
Table 3.7	Comparison of phenotype data between jaundiced babies with and without genotypic variants (Include common factors: ABO incompatibility and G6PD status)	108
Table 3.8	Comparison of phenotype data between jaundiced babies with	

and without genotypic variants (Exclude common factors:  
ABO incompatibility and G6PD status) 109

## LIST OF FIGURE

Figure		Page
Figure 1.1	Normal Pathway of bilirubin metabolism	6
Figure 1.2	Abnormal Pathway in bilirubin metabolism	7
Figure 1.3	Human UGT1 gene locus	20
Figure 1.4	Location of <i>UGT1A1</i> gene	22
Figure 2.1	Flowchart of the study design	43
Figure 2.2	Primers location	50
Figure 2.3	Standard validation using <i>HaeIII</i> puc 18	72
Figure 2.4	Standard validation using Dys 271 at 56°C	73
Figure 3.1	Sex distribution of babies enrolled in this study	85
Figure 3.2	Distrubution of babies enrolled in study according to groups and sex	86
Figure 3.3	Various type of chromatogram that indicated presence of mutation or SNPs using DHPLC for screening method	93
Figure 3.4	Distribution of mutations/polymorphisms according to the location in the gene	94
Figure 3.5	Distribution of sample with mutations or SNPs as confirmed by sequencing analysis	98
Figure 3.6	Variations appearing in the coding regions (exons) of the <i>UGT1A1</i> gene	99
Figure 3.7	Variations in the non-coding regions (introns) of the <i>UGT1A1</i> gene	100
Figure 3.8	Location of the mutations and SNPs that have been Investigated in this study ( <i>UGT1A1</i> gene)	101

## LIST OF PLATES

Plate		Page
Plate 3.1:	Genomic sample obtained from the whole blood and extracted using Qiagen blood mini kit	90
Plate 3.2:	PCR products for exon (coding region) appear in <i>UGT1A1</i> gene	91

## **LIST OF APPENDIX**

<b>Appendix</b>	<b>Page</b>
Appendix A    Borang Maklumat dan Keizinan	138
Appendix B    Patient Information	143
Appendix C    Publication and presentation	148

## LIST OF ABBREVIATIONS

$\mu\text{g}/\mu\text{l}$	:	Microgram per micro litre
$\mu\text{l}$	:	Microliter
$\mu\text{M}$	:	Micro mol
$\mu\text{mol/L}$	:	Micro mol per litre
1 TB	:	1 Timur Belakang
A	:	Adenine
$A_{260}/A_{280}$	:	Ratio of 260 absorbance/ 280 absorbance
ABO	:	Types of blood group: A, B, O and AB
ACN	:	Acetonitrile
BBB	:	Blood brain barrier
BC	:	Conjugated bilirubin/ direct bilirubin
bp	:	Base pair
<i>BRC1/BRC2</i>	:	Breast cancer type I or II
BU	:	Unconjugated bilirubin molecule/ indirect bilirubin
Buffer AE	:	Elution buffer
Buffer AW	:	Wash buffer
Buffer BL	:	Lyses buffer
Buffer EB	:	Elution buffer
Buffer PB	:	Purification buffer
B-UGT	:	Bilirubin uridine diphosphate-glucuronosyltransferase
C	:	Cytosine
c.	:	Coding number
<i>CACNLIA4</i>	:	Calcium channel gene, voltage-dependent, P/Q type alpha 1A
cDNA	:	Complementary DNA

CGH	:	Comparative genomic hybridization
CN	:	Crigler Najjar syndrome
CNI	:	Crigler Najjar syndrome type I
CNII	:	Crigler Najjar syndrome type II
CNS	:	Central nervous system
<i>COL2A1</i>	:	Collagen, type II, alpha 1
dATP	:	Deoxyadenine triphosphate
dCTP	:	Deoxycytosine triphosphate
ddH <sub>2</sub> O	:	Double distil water
ddNTP	:	Modified nucleotides
DHPLC	:	Denaturing high performance liquid chromatography
DMSO	:	Dimethylsulfoxide
DNA	:	Deoxyribonucleic acid
dNTP	:	Dinucleotide triphosphate
dsDNA	:	Double strand DNA
dTTP	:	Deoxythymine triphosphate
EDTA	:	Ethylenediaminetetraacetic acid
ER	:	Endoplasmic reticulum
G	:	Guanine
G6PD	:	Glucose-6-phosphate dehydrogenase
G71R	:	Glycine to Arginine at codon 71
GS	:	Gilbert syndrome
Hb	:	Haemoglobin
Hi-Di	:	Highly deionized
HO	:	Haem oxygenase
HPLC	:	High-performance liquid chromatography

Kb	:	Kilo base pair
kg	:	Kilogram
LOH	:	Loss of Heterozygosity
$m_1v_1$	:	Mol volume
Mer	:	Merck
MGB	:	Minor groove binder
MgCl <sub>2</sub>	:	Magnesium chloride
ml	:	Millilitre
mL/min	:	Millilitre per minute
mM	:	Micro mol
mm	:	Millimetre
mRNA	:	Messenger RNA
<i>MSX1</i>	:	Mash homobox1
n	:	Sample size
NADPH	:	Nicotinamide adenine dinucleotide phosphate
NCBI	:	National Centre of Biotechnology Informatics
ng/μl	:	Nanogram/microliter
NICU	:	Neonatal intensive care unit
nm	:	Nanometre
NNJ	:	Neonatal jaundice
O <sub>2</sub>	:	Oxygen
P229L	:	Proline to Leucine at codon 229
P229Q	:	Proline to Glutamine at codon 229
P364L	:	Proline to Leucine at codon 364
P364P	:	Proline to Proline at codon 364
PASW	:	Predictive analytic software



PCR	:	Polymerase chain reaction
<i>RB1</i>	:	Retinoblastoma protein
RBC	:	Red blood cell
RFLP	:	Restriction Fragment Length Polymorphism
RNA	:	Ribonucleic acid
rpm	:	Round per minute
S258R	:	Serine to Arginine at codon 258
SB	:	Serum bilirubin
SD	:	Standard deviation
SNP	:	Single nucleotide polymorphism
SPSS	:	Science package social software
SSCP	:	Single Strand Conformation Polymorphism
T	:	Thymine
TBE	:	Tris base EDTA
TEAA	:	Triethylammonium acetate
T412S	:	Threonine to Serine at codon 412
U	:	Uracil
U/μl	:	Unit per micro litre
UDPGT	:	Uridine diphosphoglucuronyltransferase
UGT	:	Uridine glucuronyl transferases
UGT1A1	:	Uridine glucuronosyltransferase 1A1 isoform
USA	:	United State of America
USM	:	Universiti Sains Malaysia
UV	:	Ultraviolet
ρ	:	Piko
ρmol	:	Piko mol

## LIST OF SYMBOLS

$\infty$	: Infiniti
$<$	: Less than
$>$	: More than
$^{\circ}\text{C}$	: Degree Celsius
$\sim$	: Approximately
$\text{g}$	: Gram
$\alpha$	: Alpha
$\beta$	: Beta
$\%$	: Percentage
$/$	: Or
$\pm$	: Plus minus
$P_1$	: Proportion of the G71R variant in non-jaundiced babies
$P_2$	: Proportion of the G71R variant in jaundiced babies
$Z_{\alpha}$	: Value of the standard normal distribution cutting of probability $\alpha$
$Z_{\beta}$	: Value of the standard normal distribution cutting of probability $\beta$

**PENYARINGAN *UGT1A1* GEN DAN HUBUNGKAIT GENOTIP-  
PENOTIPIK DALAM PENYAKIT KUNING DARIPADA SAMPLE BAYI  
BARU LAHIR DI KELANTAN**

**ABSTRAK**

Proses pengeluaran bilirubin adalah melalui proses glukuronidasi bilirubin yang terletak pada hati dan proses ini dimangkinakan oleh sejenis enzim iaitu Uridine glucuronosil transferase. Enzim ini dikodkan oleh gen iaitu gen *UGT1A1*. Dalam sesetengah populasi, mutasi pada gen ini akan menyebabkan berlakunya demam kuning. Walau bagaimanapun, data bagi populasi Melayu di Malaysia kurang diberi perhatian. Objektif kajian ini meliputi: mengetahui peratusan variasi pada ekson pada gen *UGT1A1* dalam populasi Melayu yang menghidap demam kuning atau tanpa demam kuning serta untuk menghubungkan penemuan dari segi genotip dan data Penotipik. Kajian keratin rentas dilakukan di Kelantan, Malaysia. Kumpulan disahkan sebagai bayi dengan demam kuning (kumpulan demam kuning) dan kumpulan bayi tanpa demam kuning (kumpulan kawalan) terlibat dalam kajian ini. Darah diambil untuk ujian genetik. DNA diekstrak daripada darah sebelum kaedah tindak balas berantai polimerase (PCR) dilakukan. Kromatografi cecair denaturasi berprestasi tinggi (DHPLC) dilakukan bagi menyaring keseluruhan ekson di dalam *UGT1A1* gen dan subjek yang dikenal pasti mengandungi bentuk ganda dua pada graf DHPLC, akan melakukan proses penjujukan DNA bagi mengesahkan kehadiran mutasi atau SNP. Data klinikal di ambil sebagai sebahagian daripada kajian yang besar yang di jalankan. Data dimasukkan melalui SPSS dan dianalisis. 286 bayi terlibat di dalam setiap kumpulan, kumpulan demam kuning dan kumpulan kawalan. Sembilan variasi telah di temui dalam kajian ini. Variasi yang paling kerap ialah

G71R pada ekson 1, yang kerap ditemui pada populasi Asia yang lain. Hanya 4 daripada variasi itu pernah dilaporkan sebagai SNPs pada populasi yang lain. Manakala variasi yang lain, di laporkan sebagai mutasi yang baru. Walaupun banyak variasi dilaporkan pada kumpulan demam kuning berbanding kumpulan kawalan, namun tiada perbezaan secara statistik antara kedua-dua kumpulan secara statistiknya adalah saling tidak berkaitan. Kajian ini juga menunjukkan tiada kaitan antara kehadiran faktor penyakit kuning pada bayi dan hemolitik antara bayi kuning dengan dan tanpa variasi dalam *UGT1A1* gen. G71R telah dikenal pasti sebagai variasi yang utama dalam *UGT1A1* gen. Keputusan kajian ini masih belum menunjukkan dengan jelas faktor menyebabkan berlakunya demam kuning.

**SCREENING OF *UGT1A1* GENE AND THE GENOTYPE-PHENOTYPE  
CORRELATIONSHIP IN NEONATAL JAUNDICE FROM A SAMPLE OF  
NEWBORNS IN KELANTAN**

**ABSTRACT**

The rate limiting step of bilirubin excretion is the glucuronidation of bilirubin in the liver, a process that is catalyzed by an enzyme, Uridine glucuronyl transferase. This enzyme is encoded by the *UGT1A1* gene. In several populations, mutations in this gene have been shown to cause neonatal jaundice. However data on the Malaysian Malay population are scanty at best. The objectives of this study included: to determine the frequency of variants in the exons of the *UGT1A1* gene in a population of term Malay neonates with jaundice and without jaundice, and to correlate the genotype finding with some phenotypic data. A cross sectional study was performed in Kelantan, Malaysia. A group of term jaundiced neonate (jaundice group) and a group of term non-jaundiced neonate (control group) were included in the study. Blood was taken for genetic testing. DNA was extracted from blood before polymerase chain reaction (PCR) was performed. Denaturing high performance liquid chromatography (DHPLC) was performed to screen the whole exons in the *UGT1A1* gene and for subjects that were identified to have a heteroduplex peak in DHPLC, sequencing was performed to confirm the mutation or SNP. Clinical data were collected as part of a larger study. Data were entered in SPSS and analyzed. Two hundred and eighteen six (286) neonates were included in each the jaundice and control groups. Nine variants have been identified in this study. The most common variant was the G71R variant in exon 1, which is common

in other Asian populations as well. Only 4 of the other variants detected in this study had been reported as SNPs in another population. Other variants appeared to be novel mutations. Even though variants were found in a higher number in the jaundice group than in the control group, the difference between the groups was statistically not significant. There were also no significant differences in severity of neonatal jaundice and in haemolysis between jaundiced neonates with and without identified variants in the *UGT1A1* gene. The G71R variant had been identified as the most common variant in the exons of the *UGT1A1* gene. The results of this study did not clearly show that this was a risk factor for neonatal jaundice.

## CHAPTER 1

### INTRODUCTION

#### 1.1 Background of the study

Neonatal jaundice (NNJ) is the most common reason for hospital admission of new born neonates. In the American population, neonatal jaundice (NNJ) was reported to occur with a frequency of 60% in full term neonates and 80% in premature neonates (Agarwal *et al.*, 2007). An earlier study suggested that the incidence of neonatal jaundice was significantly lower among Americans from African ancestry compared to African populations that are living in Africa (Kaplan *et al.*, 2008). This suggests that environmental factors may play an important role in the pathogenesis of neonatal jaundice. On the other hand, East Asians were reported to have a much higher incidence of severe neonatal jaundice than Caucasians (Yamamoto *et al.*, 2002) and this difference persisted in a population of East Asians for several generations after migration to the USA (Miller, 2008). These data suggest that the increased incidence of NNJ in East Asians is due to genetic factors. So both environmental and genetic factors may play an important role in the pathogenesis of NNJ (Ardakani *et al.*, 2007) (Kaplan M *et al.*, 2005).

Hemolytic factors such as ABO incompatibility, Rhesus incompatibility, and glucose-6-phosphate dehydrogenase (G6PD) deficiency are among the best known causes of NNJ. In Malaysia all neonates are screened for G6PD deficiency with the hope of preventing kernicterus and bilirubin encephalopathy through early detection and treatment of jaundice. Furthermore neonates with NNJ requiring hospitalization are screened for the presence of ABO incompatibility and signs of hemolysis in the blood film. Other well known causes of neonatal jaundice that are routinely looked for in the neonates with NNJ requiring therapy, include polycythaemia, birth trauma, low birth weight, prematurity, poor breast feeding (Fabris *et al.*, 2009) and a positive family history (Rennie J *et al.*, 2010). Still a lot of neonates get severe NNJ and without any of the above risk factors being clearly identified and these neonates are labelled to have idiopathic NNJ or excessive physiological NNJ.

Several genetic risks may play an important role in this group with idiopathic NNJ. Mutations in a gene known as Uridine GlucuronosylTransferase 1A1 (*UGT1A1*) gene have been investigated as a genetic factor potentially contributing to NNJ. Mutations might appear either in the enhancer region, the promoter region, exons or introns. In the Caucasian population a particular mutation in the promoter region called A (TA)<sub>7</sub>TAA mutation is proven to be a risk factor for NNJ and results in a markedly reduced expression of the enzyme (Bosma *et al.*, 1995). In some East Asian populations (Japan and South Korea) a mutation in exon 1 of the *UGT1A1* gene has been identified as a risk factor for NNJ and was associated with a close to 60% expression of the enzyme (Yamamoto *et al.*, 1998).

In Malaysia, mutations in the *UGT1A1* gene promoter and enhancer regions have been investigated and were shown to be significantly associated with NNJ (Yusoff S



*et al.*, 2010). There are few reports of small studies in the Malay population looking at mutations in exons (Sutomo *et al.*, 2004) (Yusoff *et al.*, 2006). Mutations in exons were also described by the same group of researchers in a Malay family resulting in a significant reduction of expression of the Uridine Glucuronosyltransferase (UGT) enzyme (Wada *et al.*, 2006).

The purpose of this study was to screen for mutations in the exons of the *UGT1A1* gene in a group of Malay neonates with neonatal jaundice and compare the incidence of such mutations between neonates with and without neonatal jaundice. Within the group of neonates with neonatal jaundice, an attempt was made to correlate the presence of exonic mutations with the severity of jaundice and signs of haemolysis.

In the remaining part of the introduction of this thesis, subsequently, the following topics will be covered. Neonatal jaundice will be defined after which briefly the bilirubin metabolism and some clinical aspects of neonatal jaundice will be covered. After that the author will focus in on the *UGT1A1* gene.

## **1.2 Definition of Neonatal jaundice (NNJ)**

Neonatal jaundice is a yellow discoloration of the skin, mucosal membranes and white eye (sclera) in newborn neonates. It is related to an increase in serum bilirubin levels. Jaundice becomes apparent, only if the serum bilirubin levels increase to more than 90  $\mu\text{mol/L}$  in the most cases. The jaundice usually starts in the face and progresses caudally as the serum bilirubin levels increase (Anthony and Stoll, 2007).

### **1.3 BILIRUBIN METABOLISM**

Red blood cells (RBC) are produced in bone marrow and destroyed in the spleen. When the RBC are lysed, haemoglobin (Hb), that contains heme and globin is released. The globin part is broken down into amino acids and the iron is released from the heme group. The heme group is converted to biliverdin and later to bilirubin (Maisels and Watchko, 2000). Every 1 gram of Hb produces 35mg of bilirubin (bilirubin is produced at  $8.5 \pm 2.3 \text{ mg/kg/24 hours}$ ) (Watchko, 2000).

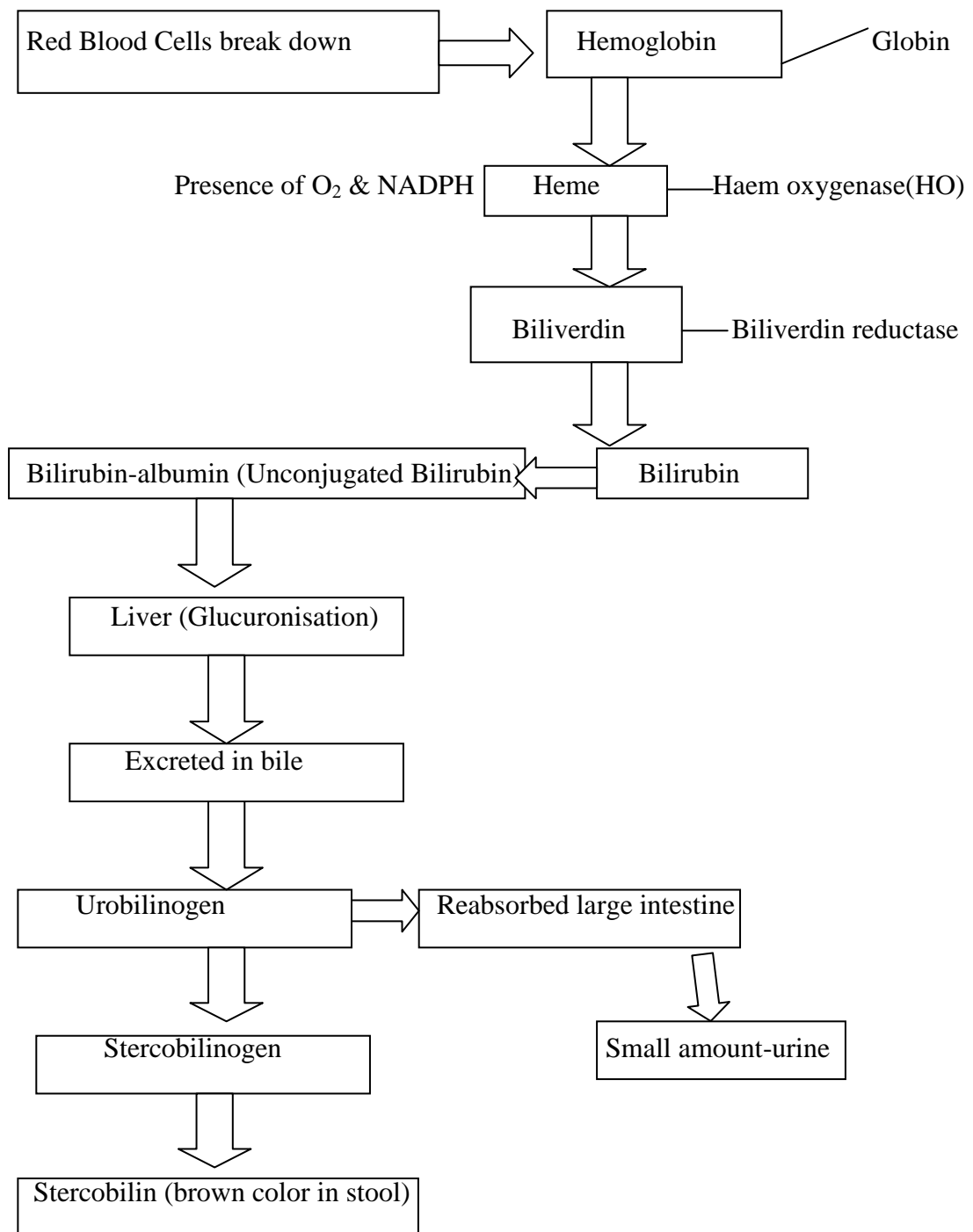
Bilirubin is a waste product of the heme metabolism. It is insoluble in water because of internal hydrogen bond interactions and it consists of four pyrrole and porphyrin rings (Fialova and Vejrazka, 2010). About 80% of the bilirubin molecules are derived from the catalysis of circulating haemoglobin (Hb). While another 20% is originated from other heme containing proteins, for example P450s and myoglobin (Tukey and Strassburg, 2000).

The bilirubin formed is unconjugated bilirubin (UB), which is lipid soluble. This molecule is transported in the plasma, bound to albumin to form bilirubin-albumin complex. When this bilirubin-albumin complex reaches to hepatocytes (liver cells), it will bind to the cell surface and bilirubin will be transported into the cell. In the liver, bilirubin will conjugate to glucuronic acid (making the molecule water soluble). This process is the rate limiting step in bilirubin excretion and is catalyzed by the uridine diphosphoglucuronyltransferase (UDPGT) enzyme. Only after conjugation it can be excreted into the bile to the small intestine. Some of conjugated bilirubin (CB) in the large intestine is metabolized to stercobilinogen before it is oxidized and changed to stercobilin which gives the brown colour to the faeces. Another part of the conjugated bilirubin is converted to urobilinogen which

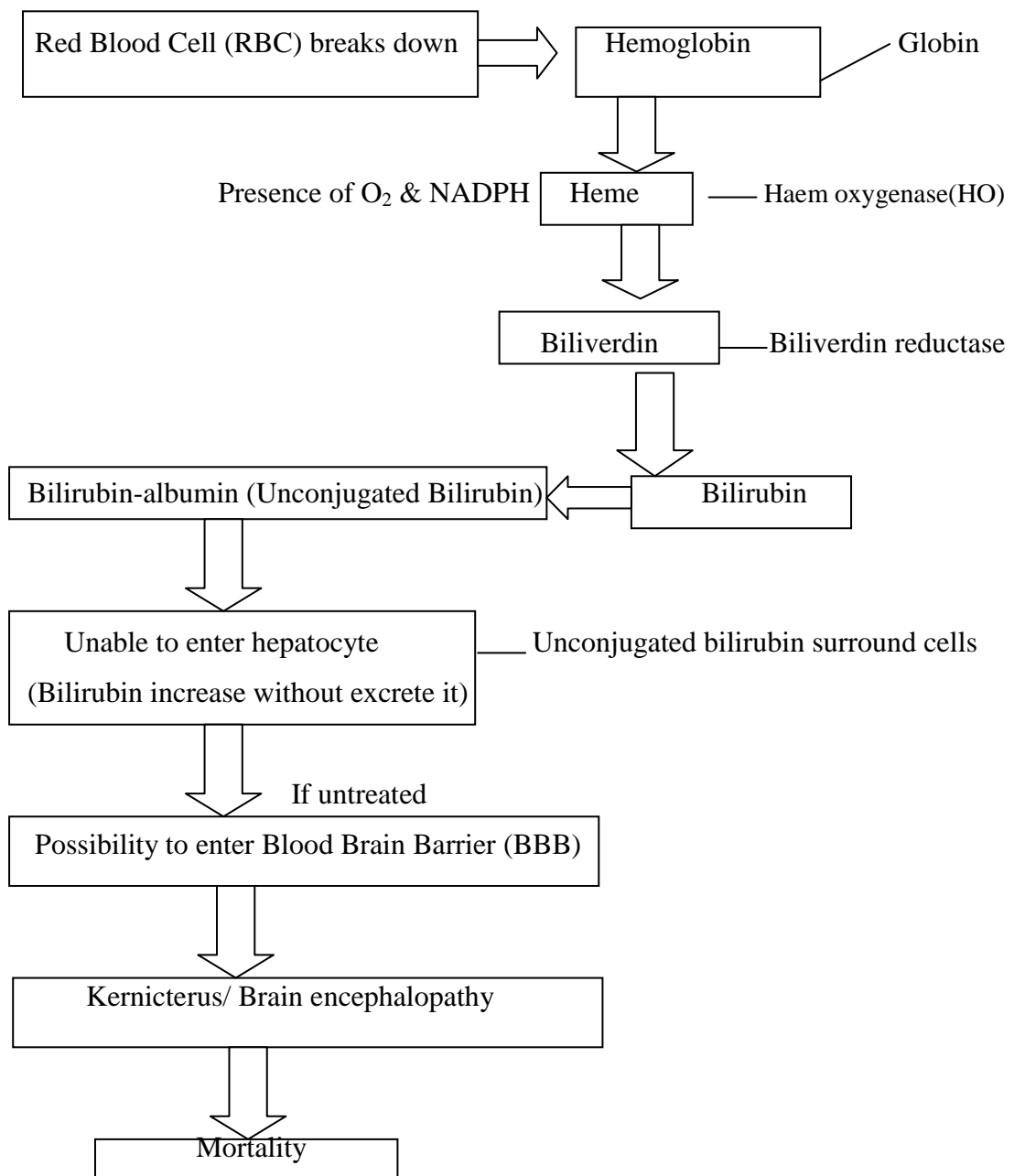
is reabsorbed and excreted in the urine in an oxidized form, called urobilin. A fraction of bilirubin from the faeces will be reabsorbed into blood via portal circulation (enterohepatic circulation). During the foetal life in the mother's womb, bilirubin is eliminated through the placenta via the umbilical cord, while during neonates, the liver will eliminate it in the conjugated form of bilirubin before it is moved to the gastrointestinal tract (Hansen, 2000).

If the unconjugated bilirubin (UB) does not excreted fast enough by the liver, the serum bilirubin will increase and bilirubin is deposited in tissues in the body leading to NNJ. This UB has the ability to cross the blood brain barrier (BBB) and cause kernicterus or bilirubin encephalopathy. Kernicterus appears when the level of serum bilirubin becomes too high and it can be confirmed during autopsy. It will give a yellow colour to the hippocampus, basal ganglia, nuclei of cerebellum and brain stem (Bhutani and L., 2005).

In most of the cases, NNJ occurs due to unconjugated bilirubin formation that is caused by immaturity of hepatocytes leading to low activity of the UDPGT. Another factor of NNJ is the short life span of RBC (between 45-90 days) in neonates as compared to adults (about 120 days) that lead to overproduction of serum bilirubin in the neonates (Moerschel *et al.*, 2008). Figure 1.2 and 1.3 shows the normal and abnormal bilirubin pathways respectively.



**Figure 1.1: Normal Pathway of bilirubin metabolism**



**Figure 1.2: Abnormal Pathway in bilirubin metabolism**

## **1.4 Clinical aspects of neonatal jaundice (NNJ)**

### **1.4.1 Unconjugated bilirubin (Indirect hyperbilirubinemia)**

Unconjugated bilirubin consists of lipid soluble or fat soluble molecules. Indirect hyperbilirubinemia occurs when the serum bilirubin level exceeds 34.2 $\mu$ mol/L.

Indirect hyperbilirubinemia leads to accumulation of free bilirubin in certain parts of the body and most importantly it can cross the blood brain barrier (BBB) and accumulate in the brain leading to kernicterus and neurological disease. It is the small fraction of unconjugated bilirubin in the plasma that is not bound to albumin (unbound bilirubin) that can move into the brain. Kernicterus was first described in 1847 by Hervieux (Juretschke, 2005) and it was observed from tissues of the brain during an autopsy where the yellow staining had appeared. Presence of endogenous or exogenous binding competitors to albumin such as certain drugs can decrease binding affinity of albumin to bilirubin and increase the risk of kernicterus.

### **1.4.2 Conjugated bilirubin (Direct hyperbilirubinemia)**

Conjugated bilirubin is identified as a water soluble molecule that moves through the bile duct to the intestine (Odell, 1980). The conjugation process involves the binding of 2 molecules of glucuronic acid to bilirubin, a process catalysed by the uridine glucuronosyl transferase (UGT). Direct hyperbilirubinaemia in the neonate is generally due to hepatocellular diseases and infectious or metabolic disorders (Klein *et al.*, 2010).

## **1.5 Common causes of neonatal jaundice**

### **1.5.1 Physiological jaundice**

Physiological jaundice is a mild form of jaundice that occurs in almost 60% of term newborns and in about 70% of preterm neonates. It appears after 24 hours (within 48 to 72 hours) of life (Saxena, 2008). The peak serum bilirubin levels are reached at the 3<sup>rd</sup> or 4<sup>th</sup> day of life for full term neonates and it disappears after 14 days of life. Usually, it is caused by a combination of increased bilirubin production due to a shorter lifespan of RBC containing foetal haemoglobin and immaturity of the liver leading to accumulation of unconjugated bilirubin. Physiological jaundice consists of two phases. In phase I serum bilirubin will rapidly increase in the first three days, while in phase II, it will decrease slowly and reaches the normal range by day 11-14 of life (Madan *et al.*, 2005). Physiological jaundice is less severe than pathological jaundice.

### **1.5.2 Pathological jaundice**

Another type of jaundice is pathological jaundice. This type of jaundice commonly appears in the first 24 hours of life (Jack, 2011) and requires treatment. It can be seen when serum bilirubin levels exceed the 95<sup>th</sup> percentile as defined in a nomogram (Kaplan *et al.*, 2006).

Pathological jaundice can be caused by haemolytic and non-haemolytic (Kaplan *et al.*, 2006) factors.

### **1.5.2.1 Haemolytic factors**

Factors associated with an increase in red blood cell (RBC) breakdown are many and include glucose-6-phosphate dehydrogenase (G6PD) deficiency, ABO incompatibility, Rhesus incompatibility, other blood group incompatibilities, polycythaemia, birth trauma, sickle cell anaemia, and other abnormalities of the RBC. In the next two subchapters a brief elaboration will be given on G6PD deficiency and ABO incompatibility.

#### **1.5.2.1 (i) Glucose-6-Phosphate Dehydrogenase (G6PD) Deficiency**

Glucose-6-phosphate dehydrogenase or G6PD is known as an RBC enzyme deficiency where the decrease of enzyme in the body might lead to haemolysis. The incidence of G6PD deficiency is higher in people from Africa, the Middle East, Arabian Peninsular, Mediterranean area and Southeast Asia including Thailand and Malaysia (Cappellini and Fiorelli, 2008). Since the 1980s, screening of G6PD in neonates has been practiced in Malaysia for early detection and treatment of neonatal jaundice and affected neonates will be observed in the ward (Ainoon *et al.*, 2003).

The reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) (Cappellini and Fiorelli, 2008) is used as a co-enzyme for G6PD which is useful to maintain the level of glutathione that will protect the RBC from oxidative stresses.

One of the well known complications of G6PD deficiency is NNJ which usually starts on day 2 to 3 of life and can be very severe (Bhutani *et al.*, 2004). In Malaysia, a study suggested a high incidence of G6PD deficiency in the Malay and Chinese populations (Singh, 1986). However it is lower in the Indian population. Its



incidence in males is much higher than in females because G6PD deficiency is an X-linked recessive disorder (Garg *et al.*, 1984).

#### **1.5.2.1 (ii) ABO Incompatibility**

ABO incompatibility cases commonly appear when a mother with blood group 'O' is having or carrying a child with blood groups either 'A' or 'B' (Mourant, 1977). In neonates with ABO incompatibility, NNJ usually appears during the first 24 to 48 hours of life (Bhutani *et al.*, 2004). However, ABO incompatibility not only occurs in neonates of mothers with 'O' blood type. It also can happen if mothers with blood group A have neonates carrying blood group B and if mothers with blood group B have neonates carrying blood group A (Iannelli, 2006).

A previous study by Kalakheti *et al.* reported that neonates with ABO incompatibility had a two times higher chance to get hyperbilirubinaemia compared to neonates with other haemolytic risk factors (Kalakheti *et al.*, 2009).

#### **1.5.2.2 Non-hemolytic factors**

In few cases, neonatal jaundice may appear due to non-hemolytic factors. These conditions include:

1. Inborn errors of metabolism such as Crigler- Najjar Syndrome types I, II and Gilbert Syndrome (Huang *et al.*, 2001). These are described in more details below.
2. Impaired bilirubin excretion leading to neonatal cholestasis, for example in Dubin-Johnson syndrome or infections such as urinary tract infection (UTI) and sepsis (MacDonald *et al.*, 2005).

3. There are two types of NNJ related to breast feeding. One is breast feeding jaundice, which is related to inadequate milk intakes early in life and this is associated with early onset jaundice. A second type is breast milk jaundice, related to a substance present in breast milk ( $3\text{-}\alpha\text{-}20\beta$  pregnanediol) that may cause NNJ in 1 to 2% of breastfed neonates. NNJ symptoms usually start on day 4 to day 7 and can last 3 to 10 weeks, or even longer. Both types are associated with an increase in enterohepatic circulation (Schmitt, 2009).

Associated factors	Effect on neonatal serum bilirubin levels		
	Increase	Decrease	No effect
Ethnicity	East Asian	African American	-
	Native American		
	Greek		
Genetic or familial	Previous sibling with jaundice	-	-
	Variant promoter or mutation of <i>UGT1A1</i> gene associated with Gilbert's syndrome		
Maternal	Older mothers	Smoking	-
	Diabetes		
	Hypertension		
	Oral contraceptive use at time of conception		
	First-trimester bleeding		
	Decreased plasma zinc level		
Drugs administered to mother	Oxytocin	Phenobarbital	Beta-adrenergic agents
	Diazepam	Meperidine	
	Epidural anesthesia	Reserpine	
	Promethazine	Aspirin	
		Chloral hydrate	
		Heroin	
		Phenytoin	
		Antipyrine	
Labor and delivery		Alcohol	
	Premature rupture of membranes	-	Fetal distress
	Forceps delivery		
	Vacuum extraction		
	Breech delivery		

Infant	Low birth weight	-	-
	Low gestation		
	Male gender		
	Delayed cord clamping		
	Elevated cord blood bilirubin level		
	Delayed meconium passage		
	Breast feeding		
	Caloric deprivation		
	Larger weight loss after birth		
	Low serum zinc and magnesium		
Drugs administered to infant	Chloral hydrate	-	-
	Pancuronium		
Other	Altitude	-	-
	Short hospital stay afterbirth		

**Table 1.1** The factors associated with neonatal jaundice (Adapted from (Maisels and Watchko, 2000)).

## **1.6 Clinical syndromes associated with mutations in the *UGT1A1* gene**

Gilbert syndrome (GS) and Crigler-Najjar syndrome (type I and type II) are the clinical syndromes which are commonly related to mutations in the *UGT1A1* gene associated with NNJ. Many studies reported that a decreased activity of the uridine diphosphoglucuronosyltransferase enzyme (UDPGT) in the liver, resulting in increases of unconjugated bilirubin levels is due to mutations in the *UGT1A1* gene (Bosma *et al.*, 1995) (Strassburg and Manns, 2000). These clinical syndromes are classified into three forms based on serum bilirubin levels (Huang *et al.*, 2001).

- The most severe form is Crigler-Najjar syndrome type I (CN I).
- Intermediate severity is seen in Crigler-Najjar syndrome type II (CN II).
- The mildest form is Gilbert syndrome.

In the Malay population reports about these clinical syndromes are scanty but mutations typically associated with Gilbert syndrome have been reported (Yusoff *et al.*, 2006).

### **1.6.1 Gilbert syndrome (GS)**

GS was discovered in 1901 by Gilbert and Lereboullet, and was described as "La cholemie simple familiale" (Sampietro and Iolascon, 1999). GS is caused by a reduction in glucuronidation activity of the *UGT1A1* enzyme in the liver by 25-50% from the normal range (Innocenti *et al.*, 2002). Mutations in the *UGT1A1* gene that may cause GS were only discovered in 1995 (Bosma *et al.*, 1995).

GS is a benign condition with a mild form of often fluctuating hyperbilirubinaemia. It might involve a single or complex locus that encodes for the enzyme, bilirubin-UDP-glucuronosyltransferase (Sampietro and Iolascon, 1999).

In Malaysia, mutations in *UGT1A1* that lead to GS were first reported in 2004 and they were found to be quite common (around 6.3%) in the studied Malay population (Sutomo R *et al.*, 2004).

Many studies showed that mutations of the *UGT1A1* gene related to GS were commonly observed in the promoter region with addition of 2 base pairs (bp) of Thymine (T) and Adenine (A) nucleotide in an area known as TATA box (Iolascon A *et al.*, 1999) (Beutler E *et al.*, 1998). The normal repetition of TA is 6 and an addition of TA as seen in the A (TA)<sub>7</sub>TAA mutation, also known as *UGT1A1*\*28, might lead to Gilbert syndrome. The addition of TA causes impairment of proper message transcription that can cause a decrease of *UGT1A1* activity in the liver (Bosma *et al.*, 1995).

A study from Scotland also suggested that GS not only contributed to prolonged jaundice in breast fed infants but also led to pharmacological variations in drug glucuronidation and sometimes unexpected toxicity from therapeutic agents (Burchell and Hume, 1999).

In Liverpool, GS had been studied as the most inherited metabolic liver disorder (around 6%) in a Caucasian population (Owens and Evans, 1975). This report had been supported that the A (TA)<sub>7</sub>TAA mutation in the promoter region is the most common cause of GS in Caucasians (Beutler *et al.*, 1998) compare to Asian countries. In Asia, mainly in East Asians countries, it has been proven to be more

commonly due to a mutation in exon 1 known as the G71R mutation (Maruo *et al.*, 1999b).

### **1.6.2 Crigler-Najjar Syndrome (CN)**

Crigler-Najjar syndrome (CN) was discovered in 1952 by 2 researchers, Crigler and Najjar. CN is also known as congenital non-obstructive non-hemolytic hyperbilirubinemia (John F.Crigler, 1952)

Mutations in the *UGT1A1* gene leading to unconjugated bilirubin in CN was first reported by Ritter et al in 1992 (Ritter *et al.*, 1992). CN had been classified into two types known as Crigler-Najjar Syndrome type I (CN I), discovered by Crigler and Najjar and Crigler-Najjar Syndrome type II (CN II) discovered by Arias, based on the level of serum bilirubin and the treatments (Arias, 1962).

CN is marked by partial or total deficiency of the glucuronyl-transferase enzyme and might cause fatal hyperbilirubinaemia in neonates if untreated. In severe cases, this syndrome may lead to brain damage (kernicterus) to neonates and early screening needs to be performed to avoid it. CN can be caused by mutations in intron (non-coding) or exon (coding) regions of the *UGT1A1* gene.

#### **1.6.2 (i) Crigler-Najjar Syndrome Type I (CN I)**

Crigler Najjar syndrome type I or CN I is inherited in an autosomal recessive way and is a rare syndrome (Arias *et al.*, 1969). Studies suggested that CN I is caused by a complete absence of UDPGT enzyme activity toward bilirubin in the hepatocytes.

CN I occurs in 1:1,000,000 newborns and can be observed in the first day of life with high levels of serum bilirubin (Labrune, 2004). Neonates with CN I do not

respond to phenobarbital treatment. CN I can be caused by several types of mutation such as homozygous nonsense, frameshift or homozygous missense mutation (Kadakol *et al.*, 2000). CN I is mainly associated with the mutations in exons 2-5 that might affect the termination codon (Clarke *et al.*, 1997) and premature truncation of the *UGT1A1* gene (Kadakol *et al.*, 2001). Mutation in CN I was first reported in 1992 (Ritter *et al.*, 1992) and CN I can be diagnosed using high-performance liquid chromatography (HPLC) for analysis of the bile or tissue enzyme assay from liver biopsy. A study in 2007 showed that CN I also often appeared in the children from consanguineous parents as described in Slovakia's population (Zmetakova *et al.*, 2007).

#### **1.6.2 (ii) Crigler-Najjar Syndrome Type II (CN II)**

Crigler- Najjar syndrome type II or CN II is also mainly inherited as an autosomal recessive trait (Arias *et al.*, 1969) even though reports of autosomal dominant inheritance are available (Arias, 1962). The serum bilirubin level is markedly elevated but less than in CN I (Jansen, 1999).

Enzyme activity is severely reduced in CN II but it can be induced following phenobarbital administration. CN II rarely causes damage to the central nervous system (CNS) and the majority of the patients can survive until adulthood without any complications. CN II has been more frequently reported in Asian populations as compared to other populations (Yamamoto K *et al.*, 1998).

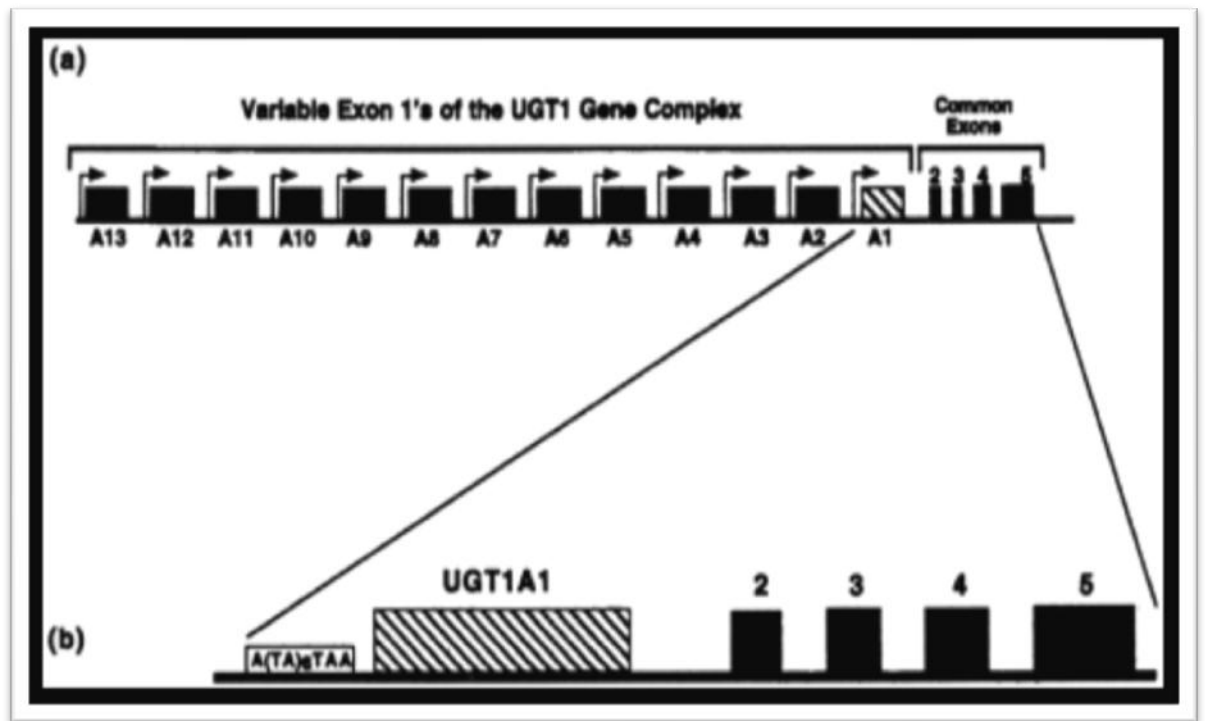


## **1.7 Uridine Diphosphoglucuronate Glucuronosyl Transferase (UDPGT) 1A gene**

UDPGT is an enzyme found in many cells in the human body such as the liver, intestines, kidneys, lungs and adrenals. Uridine glucuronyl transferases (UGT) consist of a large superfamily and are classified into two major isoforms based on the nucleotide sequence for the identification of their own isoforms, UGT1 and UGT2. These isoforms provide glucuronidation of bilirubin, oestriol, oestradiol, quinols and phenols. It represents a super family of microsomal membrane-bound enzymes that catalyze conjugation that is not only limited to bilirubin (Kaplan and Hammerman, 2005).

UGT1 encodes a biochemical reaction for the conjugation of glucuronic acid. This isoform also mediates glucuronidation of other aglycone substrates in *UGT1A* locus. *UGT1A1* is the only isoform that contributes substantially to the bilirubin glucuronidation transferase (Kaplan and Hammerman, 2005).

The length of the gene encoding the UGT1A family is 218kb, consisting of 13 variable isoforms in exon 1 and all these isoforms are spliced into 4 common exons (exon 2 to exon 5). From the 13 variable isoforms, only 9 isoforms encode for protein (1A1, 1A3, 1A4, 1A5, 1A6, 1A7, 1A8, 1A9, and 1A10) while another 4 isoforms are pseudogenes, not functionally important in the UGT1A family (1A2, 1A11, 1A12 and 1A13). Only variable exon A1 is important in bilirubin conjugation and the others play a role in the detoxification of a diverse range of chemical substances (Kaplan and Hammerman, 2005). Exons 2 to 5 are responsible in specific signal and transcription processing splices of mRNA from single variable exons to the common exons.



**Figure 1.3:** Human UGT1 gene locus.

(a) Schematic representation of the genomic structure of the UGT gene complex.

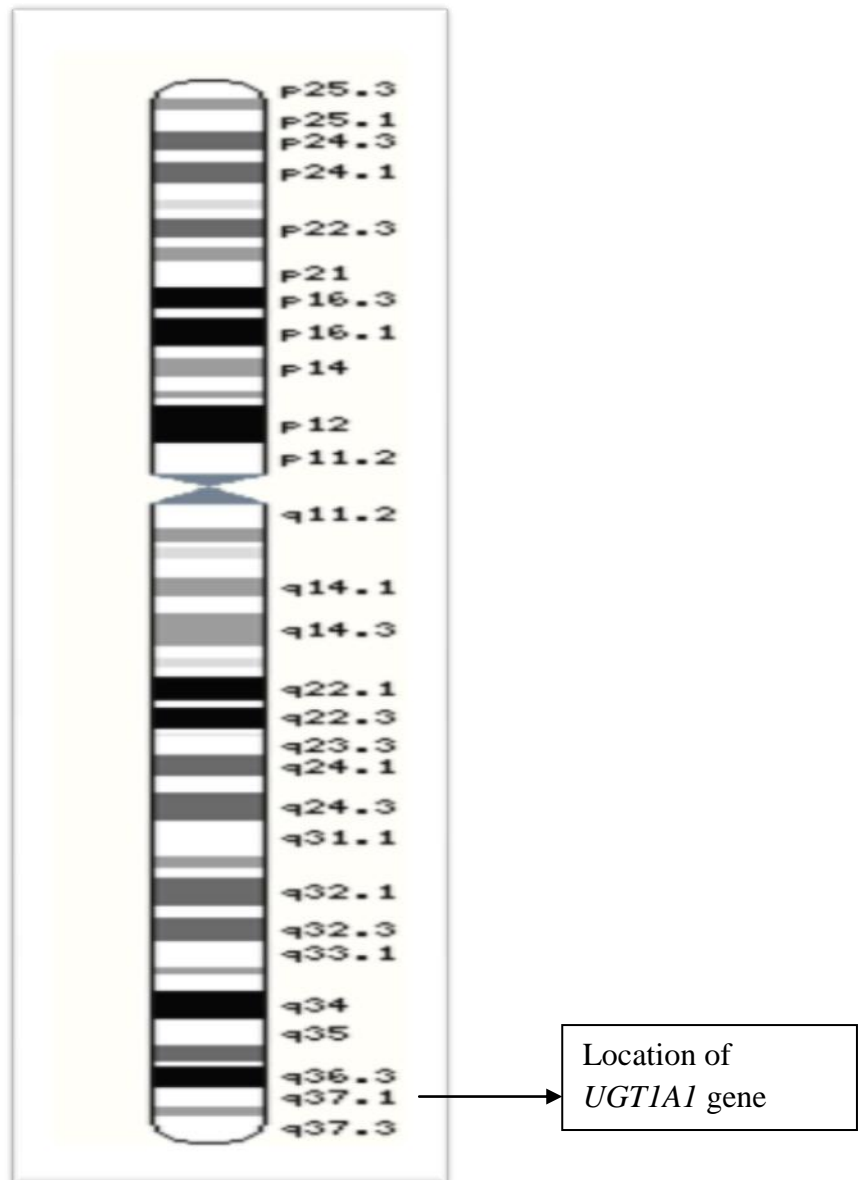
(b) Exploded view of exon 1A1 and common exons 2-5 of the gene complex that have been identified as sites for genetic mutations associated with absent or decreased of UGT activity that cause deficiencies of bilirubin conjugation. Used with permission from Clarke (Clarke *et al.*, 1997)

### **1.8 Bilirubin Uridine Diphosphate-Glucuronosyltransferase 1A1 (*UGT1A1*) gene**

The bilirubin uridine diphosphate-glucuronosyltransferase 1A isoform 1 or *UGT1A1* gene is located in the q arm of chromosome 2, 2q37.1 (see figure 1.4). Other names for the *UGT1A1* gene include GNT1, Uridine Diphosphate Glucuronosyltransferase (UDPGT) and HUG-BR1. It consists of 5 exons (1-5, where exon 1 is spliced to exon 2-5) and the specific promoter region. This gene is located at the 3' end of the *UGT1A* locus (Costa *et al.*, 2006).

The common exons in UGT1A family (exons 2-5) are located at 3'end which encode carboxylterminal domain for all *UGT* isoforms and bind with UDP-glucuronic acids. Each isoform in the UGT1A family has the unique exon in exon 1 and also their own promoter which is located in the 5' N-terminal domain. The *UGT1A1* gene has an approximate length of about 13 kb (13,027bp) which is not including other genes that overlap in the UGT1A family transcripts. Bilirubin uridine diphosphate-glucuronosyltransferase (B-UGT) is the key enzyme for bilirubin metabolism, and decreasing of *UGT1A1* gene activity leads to unconjugated hyperbilirubinemia that may result in Gilbert Syndrome or Crigler-Najjar Syndrome. Mutations that appear in exons and promoter of the *UGT1A1* gene may cause structural or functional abnormalities of the enzyme, causing impaired bilirubin conjugation, leading to hyperbilirubinemia (Kaplan *et al.*, 2003).

Mutations of the *UGT1A1* gene may affect the bilirubin glucuronidation activity that is leading to these inherited disorders. Mutation in the promoter region reduces the *UGT1A1* gene expression while mutation in the coding region cause dysfunction of the *UGT1A1* gene and also an abnormality of the gene structure (Sun *et al.*, 2007).



**Figure 1.4** Location of *UGT1A1* gene in chromosome 2 (2q37.1)

Adapted from:

[http://www.ensembl.org/Homo\\_sapiens/Location/Chromosome?g=ENSG00000241635;r=2:234669368-234682419;t=ENST00000305208](http://www.ensembl.org/Homo_sapiens/Location/Chromosome?g=ENSG00000241635;r=2:234669368-234682419;t=ENST00000305208)

### 1.9 Mutations in the *UGT1A1* gene

Mutations of the *UGT1A1* gene can be classified into three groups:

1. Mutations that result in reduced production of the enzyme (Guillemette C., 2003).
2. Mutations that give rise to the synthesis of an enzyme that is structurally and/or functionally abnormal (Sampietro M *et al.*, 2003).
3. Mutations which completely abolish the expression of the affected allele (Sampietro M *et al.*, 1999).

Mutation is defined as a permanent change in the genome, either in DNA or RNA sequence which cause changes in amino acids or proteins related to that particular gene. Mutations may occur due to radiation, viruses and mutagenic chemicals which cause errors during meiosis or DNA replication (Bertram, 2001).

Mutations in the *UGT1A1* gene occur in the coding region, known as exon and in the non coding region, known as intron. Since in the middle of 1980s more prevalence of mutations in the *UGT1A1* gene had been discovered and mutation that is responsible for Gilbert syndrome was first described in 1995 by Aono et al (Aono *et al.*, 1995).

As discussed above (section on Gilbert Syndrome), an important mutation in Caucasians is located in the promoter region. Association of mutations in the promoter region and enhancer region have been found to be significant risk factors for NNJ (Bosma *et al.*, 1995) (Yamamoto *et al.*, 2002) and recently this has also

been confirmed in a Malay population (Yusoff S, 2010). The focus of the current study is however on the exonic region of the *UGT1A1* gene.

One of the exonic mutations that seems to play a real important role in neonatal jaundice in Asians (Long *et al.*, 2011) is the G71R mutation. It is a common mutation of the *UGT1A1* gene and has been widely reported in East Asian countries such as Taiwan, China and Japan.

In Southeast Asian populations, this mutation of the *UGT1A1* gene was believed to be first described in a Thai population (Sutomo *et al.*, 2002). Other reports about Southeast Asia include studies done in Singapore (Zhou *et al.*, 2009), Indonesia (Sutomo *et al.*, 2004) and Malaysia (Yusoff *et al.*, 2006) (Boo *et al.*, 2009). The findings in each of these studies are compared to the findings in the current study in the discussion part of this thesis. The G71R mutation is located in exon 1 of the *UGT1A1* gene and comprises of a substitution of Guanine to Adenine (G to A) at nucleotide 211. It causes a change of amino acids from glycine to arginine at codon 71 (G71R). The G71R mutation seems to be rare in Caucasian populations (Narter *et al.*, 2011) and because of the high prevalence of this mutation, in Asian population, it had been considered as polymorphism for Asian people (Takeuchi *et al.*, 2004).

A study in Japan showed that the G71R mutation, either in heterozygous or homozygous trait, may decrease the enzyme activity by 32.2% and 60.2% respectively using in- vitro expression (Yamamoto *et al.*, 1998). This condition may

lead from mild to moderate delay of bilirubin elimination in human (Sun *et al.*, 2007).

Another major mutation in Asian populations, mainly in the Japanese population, related to NNJ is the P229Q or Pro229Gln mutation which is also located at exon 1 of the *UGT1A1* gene. This mutation causes substitution of nucleotide Cytosine (C) to Adenine (A) at nucleotide 686 (Saeki *et al.*, 2003). There are studies suggesting that a combination of mutations (TATA box with G71R or P229Q) increases the serum bilirubin levels in neonates (Kamisako, 2004).

However, a study in Taiwan suggested that the combination of promoter or G71R and P229Q might not affect the serum bilirubin level significantly in neonates (Hsieh *et al.*, 2001).

As mutation at the same codon has also been observed in African-American population but a different nucleotide was involved. It caused a change of nucleotide from Cytosine (C) to Thymine (T) and is known as P229L, causing an amino acid change from Proline to Leucine (Kaniwa *et al.*, 2005b).

Besides G71R and P229Q mutations in exon 1, the Y486D in exon 5 of the *UGT1A1* gene had been suggested to contribute in the development of neonatal jaundice (Takeuchi *et al.*, 2004). The Y486D mutation causes a substitution of nucleotides (from Thymine (T) to Guanine (G) at position number 1456, leading to an amino acid change, Tyr486Asp. This results in a decrease of 7% of the *UGT1A1* gene enzyme function (Wu *et al.*, 2008).